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The Mechanism of Glucocorticoid Effects in Fibroblasts

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In vivo, the glucocorticoid steroids inhibit fibroblast growth and the production of specific cell products such as collagen and mucopolysaccharide. Depending on the source of the fibroblasts and their growth conditions, several responses can be observed in cultured cells; the proliferation of L cells is inhibited, the growth of synovial fibroblasts is usually stimulated, and some cultured fibroblasts do not respond. The inhibition of L cell growth is preceded by alterations in the rates of hexose and amino acid transport. As in other cell types, there is evidence that the effects of glucocorticoids in fibroblasts are mediated via the induction of new protein synthesis. The specific induced proteins responsible for inhibiting the growth of mouse L929 fibroblasts (or any other cell type) have not been identified. It is possible that glucocorticoids induce the synthesis of a few proteins that, by virtue of their ability to modify (either during or after translation) other proteins involved in several key cellular events, are able to compromise multiple systems and produce growth inhibition. Glucocorticoid-mediated alteration of protein phosphorylation could account for the pleiotropic nature of the hormone effect.

To cause a glucocorticoid-specific response in the cell, these hormones must first bind to a cellular receptor. A significant amount of evidence suggests (a) that glucocorticoid receptors may undergo an energy-dependent cycling in the cell and (b) that a single receptor may be able to participate several times in the process of steroid binding and gene activation. Maximal physiological response to, and consequently the clinical response achieved with, a glucocorticoid is usually a function of the extent to which the hormone can be bound in a specific manner. Investigators have proposed that the specific glucocorticoid-binding capacity of a cell is determined by a phosphorylation-dephosphorylation mechanism.

Glucocorticoids differ from other steroid hormones in that they produce effects in almost all tissues and cell types. The nature of the physiological response varies according to the cell or system being examined. Munck and Leung in an excellent comprehensive review [1] have recently summarized the wide variety of physiological and biochemical effects produced by glucocorticoids. In general, the physiological effects of glucocorticoids in fibroblasts are antianabolic. That is, these drugs inhibit growth and the production of important cell products such as collagen [2] and mucopolysaccharide [3]. Even within the 1 cell type, however, the physiological response is not constant. The growth rate of some fibroblasts maintained in culture is actually stimulated by exposure to low concentrations of glucocorticoid, and in a few systems no alteration in growth rate has been observed. Thus, it is appropriate that I begin this discussion with a short review of the effect of glucocorticoids on fibroblast growth.

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Effects of Glucocorticoids on Fibroblast Growth

Glucocorticoids are capable of inhibiting the processes of wound healing and fibrosis. In early efforts to understand the profound effects of this class of hormones on connective tissue, several investigators [4,5] established that systemically administered glucocorticoids can inhibit the growth of fibroblasts in model would repair systems. Although changes in connective tissue were not observed by Baker and Whitaker [6,7] in the first studies on glucocorticoid effects after local administration, Castor and Baker [8] subsequently demonstrated a considerable reduction in the number of dermal fibroblasts in areas of rat skin exposed to topically applied drug. Later, it was shown that cortisol and other glucocorticoids can inhibit the rate of growth of fibroblasts in culture [9], a finding that confirmed earlier conclusions that the effect was independent of the actions of these hormones on other cell types and on the vasculature. The earliest studies showing an inhibition of fibroblast growth in vitro employed exceedingly high drug concentrations [10–12], and it was not until the work of Ruhmann and Berliner [13] in 1965 that an unequivocal demonstration of the growth inhibitory effect was accomplished in a readily manipulated, well-characterized fibroblast line growing in vitro. These workers carried out extensive stereospecificity studies in L 929 mouse fibroblasts (this line was originally derived from subcutaneous connective tissue of a C3H/An mouse) and demonstrated that the activity of steroids in suppressing L cell growth reflects their clinical efficacy as anti-inflammatory agents [13,14]. The major exceptions are the 11-keto compounds (cortisone, prednisone) that do not inhibit growth in L cells because they are not converted to the active 11-hydroxy form [14].

In general, steroids inhibit the growth rate of L cells [9,15,16] but do not cause the cytolysis that occurs in some cell types (e.g., thymic lymphocytes [17]). Even after several days of constant exposure to maximum inhibitory concentrations of glucocorticoid, growth resumes on withdrawal of the drug [16,18]. The major exception to these observations may be the work of Rhumann and Berliner [13]; their observation that large decreases in cell number occurred within 3 to 5 days after introduction of potent steroids into L cell monolayer cultures suggests that considerable cell killing occurred in their experiments.

A definite change in cell structure accompanies the steroid effect. Steroid-treated L cells are larger, flatter, less densely packed, and often more polygonal and epithelial in appearance than controls [19,20]. Some investigators have speculated that this morphological change may represent a phenotypic reversion from a "transformed, or tumor cell-like state to a more tightly regulated growth state" [20].

Glucocorticoids inhibit DNA synthesis and growth in primary chick embryo fibroblast cultures, although they have no effect if the cells have been transformed with Rous sarcoma virus [21]. Cultured fibroblasts do not always respond to glucocorticoids with a negative growth effect. The growth of SM-C1 fibroblasts (derived from a rat carrageenan granuloma) is not altered, although cell functions such as secretion of collagen and glycosaminoglycans are markedly decreased [22]. Glucocorticoids also inhibit the production of these intercellular substances in fibroblasts cultured from human synovial tissue, but these cells usually respond with a moderate increase in growth rate [2,3,23,24]. Considerable interest has focused on the growth-promoting effect of glucocorticoids in 3T3 mouse

fibroblasts. The 3T3 lines (derived from disaggregated mouse embryos) are density-inhibited cell systems that have been used as a model in studies on growth regulation and transformation. Thrash and Cunningham observed that the addition of glucocorticoid to density-inhibited 3T3 cells was followed by the initiation of cell growth [25,26]. The effect is specific for glucocorticoids, and the steroids have no effect in polyoma- or SV40-transformed 3T3 lines. Although it is not clear from this work whether the growth stimulation was a direct steroid effect or whether the glucocorticoid may have "permitted" the growth-stimulating action of factors in serum, results from other studies strongly suggest the latter mechanism.

Armelin [27] showed that, although hydrocortisone has no growth-promoting effect of its own in 3T3 cultures, growth stimulated by pituitary extracts is greatly enhanced by this steroid. Using different 3T3 lines, Gospodarowicz and others [28–30] confirmed that glucocorticoids alone do not stimulate the initiation of DNA synthesis or cell proliferation in 3T3 cultures, and they showed that the steroids potentiate the effect of fibroblast growth factor (FGF). The FGF is a polypeptide with a molecular weight of 13,300, that has been purified from bovine pituitary gland [31]. In the presence of a low concentration of dexamethasone, FGF can sustain multiple divisions of 3T3 fibroblasts in serum-starved cultures (although it should be made clear that the FGF effect is not specific for these cells or even for fibroblasts since it can act as a mitogen for a variety of cell types from murine, bovine and human sources [31]). It has been noted that when 3T3 cells are permitted to grow to high density by exposure to FGF in the presence of glucocorticoid, they assume the appearance of transformed cells [32].

When prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is added to quiescent 3T3 fibroblast cultures, DNA synthesis and growth are initiated [33]. This stimulation is inhibited by low concentrations of glucocorticoids [34]. Thus the same concentrations of hydrocortisone can increase the rate of DNA synthesis induced by FGF [28,29] and decrease the rate induced by $PGF_{2\alpha}$ in the same Swiss mouse 3T3 fibroblast cultures.

Reports regarding the effect of glucocorticoid on human skin cells in culture have been somewhat conflicting. Thrash and Cunningham [25] reported that early passage diploid human foreskin fibroblasts were stimulated by cortisol. Gospodarowicz and Moran [35], however, found no growth stimulation by dexamethasone alone and no potentiation of the FGF effect in similar cultures. Kamely and Rudland [36] examined 2 cell lines obtained from human skin biopsy specimens, and again glucocorticoid neither sustained growth nor potentiated the effect of FGF.

Summary. Glucocorticoids administered to animals either systemically or topically can depress fibroblast growth and function. A similar growth-inhibiting effect occurs in L 929 mouse fibroblasts in culture. The relative potencies of steroids in inhibiting L cell growth correlates well with their topical anti-inflammatory activity [37]. Several types of fibroblasts in culture do not respond to glucocorticoids with a decrease in growth rate. In the case of some density-inhibited fibroblasts, the glucocorticoids potentiate the effect of polypeptide growth factors but they usually do not affect the growth rate when they are present alone. When these cells are transformed, the glucocorticoids no longer affect their growth. The growth of fibroblasts cultured from human synovial tissue is stimulated, but this effect may reflect an ability of these steroids to enhance the growth-promoting effects of serum factors. In the remainder of this paper I will review studies on the mechanism of action of glucocorticoids in fibroblasts.

Biochemical Events Inhibited by Glucocorticoids in Mouse Fibroblasts

Initial studies on the biochemical effects of glucocorticoids in L cells focused on possible alterations in the rates of protein and nucleic acid synthesis. When L cells growing in log phase in monolayer culture were exposed to maximally effective concentrations of glucocorticoid (e.g., 5×10^{-7} M fluocinolone acetonide), an inhibition of the rate of incorporation of thymidine into DNA was evident within about 6 hr [15]. After 24 hr of exposure to hormone, incorporation of thymidine was inhibited by about 50% and that of uridine by about 25% (Fig 1). Protein synthesis, as estimated by the incorporation of radiolabeled leucine into the acid-insoluble fraction, was not inhibited. Indeed, the incorporation of leucine expressed on a per cell basis was minimally stimulated [15]. Seifert and Hilz [16] assayed thymidine incorporation rates in suspension cultures of L cells over a period of several days of exposure to cortisol. Maximum inhibition of thymidine incorporation was about 70% and was achieved in 24 hr. Incorporation of radiolabeled proline into cold acid-insoluble material was increased in cells exposed to steroid. The observation (made under both culture conditions) that thymidine incorporation was never completely inhibited while cell growth was virtually stopped is consistent with the fact that DNA content per cell increased about 1.5-fold in the experiments of Seifert and Hilz [16].

Nucleic acid synthesis has also been studied in intact nuclei isolated from L cells exposed to fluocinolone acetonide for 12 hr prior to harvest [38]. It was demonstrated that incorporation of ribo- or deoxyribonucleoside triphosphates into cold acid-insoluble material by these nuclear preparations is actinomycin-D-sensitive and requires the presence of all four complementary nucleotides. The products of [^{3}H]dATP and [^{3}H]ATP incorporation were chemically identified as DNA and RNA, respectively. The DNA was of low molecular weight and did not sediment with bulk DNA in alkaline sucrose gradients. In this system, the ability of nuclei from cortisol-treated cells to incorporate nucleoside triphosphates into both DNA and RNA was inhibited about 25% relative to controls [38]. Because the substrates were provided in the triphosphorylated form, these results suggest that within 12 hr the steroid affected components of the nucleic-acid-synthesizing systems. Thus, all of the inhibition of radiolabeled nucleoside incorporation observed in intact cells was probably not due to effects on precursor uptake or conversion to triphosphates.

Inhibition of nucleic acid synthesis is a late effect of the hormone, and it is not clear from these studies the extent to which it contributes to the observed growth inhibition. Prior to effects on nucleic acid synthesis, the uptake of hexose and amino acids into the cell was inhibited. Co-workers and I examined the effect of steroids on the uptake of radiolabeled galactose and 2-deoxyglucose in L cells [39]. Galactose is taken up by L cells and converted to galactose-1-phosphate and

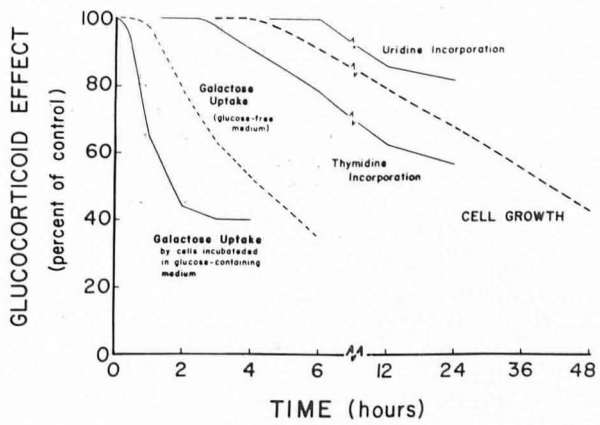

FIG 1. Time course of inhibition of hexose uptake, nucleoside incorporation, and growth caused by glucocorticoid in L 929 mouse fibroblast cultures (data for galactose uptake taken from Gray, Pratt, and Aronow [39]; data for nucleoside incorporation and growth taken from Pratt and Aronow [15]). Galactose uptake was used as a measure of the effect of glucocorticoids on the rate of hexose transport. The rates of galactose uptake or nucleoside incorporation are expressed as percentages of control rates assayed in untreated cultures. I obtained the curve defining inhibition of cell growth by counting the number of cells per glucocorticoid-treated culture and expressing this value as a percent of the cell count in untreated controls.

uridine diphosphogalactose, but these cells lack the enzyme uridine diphosphogalactose-4-epimerase [40] and galactose cannot be utilized as an energy source [41]. Because the uptake mechanism for galactose and 2-deoxyglucose, another non-energy-yielding hexose, is shared with glucose [39], we used these compounds to measure hexose uptake and phosphorylation. (It should be noted, however, that glucose is taken up by 2 mechanisms in these cells and the one that is apparently not shared with galactose could be physiologically more important.) When L cells were suspended in an isotonic salt solution in the absence of glucose, inhibition of radiolabeled galactose uptake was not apparent until 1 to 2 hr after addition of glucocorticoid (Fig 1). When glucose was present in the suspension solution, inhibition of radiolabeled galactose uptake was observed much earlier (within 30 min to 1 hr). This enhancement of the steroid effect by glucose required metabolism. When cells were preincubated for 90 min in the absence of glucose but radiolabeled galactose uptake was measured in the presence of glucose, the steroid-mediated inhibition was the same as in glucose-free controls. When glucose was present during the preincubation period, however, inhibition of galactose uptake was about 1.7-fold that observed in control samples. The fact that cells preincubated with glucose were more sensitive to the steroid effect than controls incubated in the absence of substrate suggests that active cell metabolism is necessary for rapid expression of the hormone effect. It was not possible to measure 3-0-methylglucose uptake rates in L cells, and it was not clearly demonstrated that the inhibition of hexose uptake represented an effect on transmembrane passage rather than phosphorylation.

On the basis of detailed observation of thymic lymphocytes in which inhibition of glucose uptake was seen within 15 to 20 min after addition of glucocorticoid [42,43], Munck suggested that the inhibition of glucose uptake is an essential step in the catabolic actions of glucocorticoids in these cells [44]. In L cells, growth supported by high concentrations of fructose (this hexose is metabolized but it does not interact with the uptake processes for glucose or galactose) is as sensitive to inhibition as growth supported by glucose [39]. This fact argues against the proposal that inhibition of hexose transport is absolutely necessary for the growth-inhibiting effect of glucocorticoids in this system.

Glucocorticoids have also been shown to inhibit the rate of uptake of α-aminosobutyric acid (AIB) in mouse fibroblasts [39]. This is an amino acid that is not utilized by L cells but that is actively transported into them by a process shared with some of the naturally occurring L-amino acids [45]. Inhibition of hexose and AID uptake has been demonstrated in preparations of mouse skin exposed to glucocorticoids in vitro [46–48].

As described above, general protein synthesis in L cells is not inhibited by glucocorticoids [15], but it is possible that selective inhibition of the synthesis of some individual proteins may occur, and that this inhibition may be of some consequence to the growth-inhibiting effect. Plasminogen activator is a protein that may play an important role in permitting rapid growth of some cultured cells, and the plasminogen activator activity of L cells is markedly decreased on exposure to 10^{-7} M dexamethasone [20]. Wong and Aronow [49] have recently reported selective inhibition of incorporation of radioactive amino acids into a lysine-rich histone component identified in L cell nuclei. It is not clear whether the finding reflects decreased synthesis of this protein fraction or some other mechanism, and its relationship to the glucocorticoid-mediated effects described above is unknown. The affected component accounts for less than 1% of the total histones, and it appears to be lost in a first-order manner; about 50% remains after 4 hr [49].

Summary. A number of factors may contribute to the growth-inhibiting effect of glucocorticoids in cultured mouse fibroblasts. As in several other cell types, an inhibition of hexose uptake is apparent prior to inhibition of nucleic acid synthesis. The mechanisms of inhibition of transport and nucleic acid synthesis are not known. The kinetics of inhibition of hexose uptake and nucleic acid synthesis is presented in Figure 1. The onset of these inhibitory effects is later, and the time course of their development is slower than those observed in lymphocytes in which the steroid effect is cytolytic [43, 50].

Fibroblasts and the Model of Steroid Hormone Action

Although work on the mechanism of glucocorticoid action has not advanced as far as that of sex hormones, there are enough observations common to all steroid systems for a general model of steroid action to have been developed. In Fig 2, steps 1 through 3 depict the entry of the steroid into the cell, its binding to a specific receptor protein located in the cytoplasm, and the conversion of the steroid-receptor complex to a form (RS^n) capable of binding to an acceptor site in the nucleus. (These events will be considered in subsequent sections of this review.) The details of the association of the RS^n complex with chromatin have not yet been defined, but evidence obtained in several glucocorticoid-responsive systems is consistent with the general hypothesis that nuclear binding of the complex in some way causes the production of specific mRNA and the consequent synthesis of new proteins. The physiological effect produced by the glucocorticoids depends on the nature of the induced proteins and that in turn is a function of the cell type being examined, that is, virtually all cells contain glucocorticoid receptors and follow the same pattern of events shown in the model, but the types of mRNA that are induced, and consequently the physiological response of the cell, are determined by differentiation.

The most difficult portion of the model to examine with respect to the growth-inhibiting action of the glucocorticoids may well prove to be the identification of the induced proteins that are ultimately responsible for the antianabolic effect. No protein (or set of proteins) that could be considered an initiator of growth inhibition (or lethal protein in the case of those cell types that are killed) has been identified in any type of glucocorticoid-responsive cell. Observations from the laboratories of Munck [43] and White [51] suggest the existence of a class of glucocorticoid-induced inhibitory proteins in thymic lymphocytes. It has been demonstrated that the inhibition of glucose transport occurring in thymocytes 15 to 20 min after exposure to glucocorticoid is blocked if actinomycin D is added along

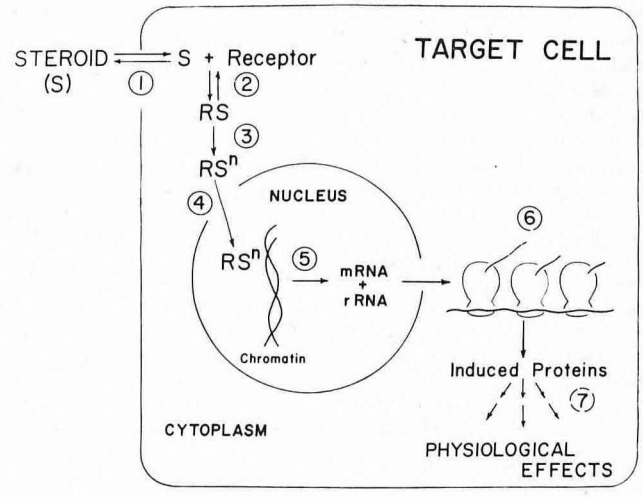

FIG 2. Model of glucocorticoid action. After entering the cell (*step 1*), the steroid binds (*step 2*) in a stereospecific, noncovalent manner to a soluble protein receptor (*R*) located in the cytoplasm and thus forms a steroid-receptor complex (*RS*). The complex then undergoes a temperature-dependent transformation (*step 3*) to a form (RS^n) capable of binding to acceptor sites in the cell nucleus (*step 4*). The association of the RS^n complex with chromatin in some way causes the synthesis of specific mRNAs (*step 5*) and consequent new protein synthesis (*step 6*). The newly synthesized proteins produce the cellular alterations that mediate the gross physiological effect. In the case of L cells, there is growth inhibition.

with the steroid [52], but addition of actinomycin D 5 min after addition of cortisol does not prevent the steroid effect. Inhibitors of protein synthesis (cycloheximide and puromycin) block the glucocorticoid-mediated decrease in hexose transport if they are present from 15 to 30 min after the introduction of the steroid but not if they are only present prior to 15 min [43]. Observations of this type have prompted the suggestion that glucocorticoids rapidly induce the transcription of an mRNA that in turn directs the synthesis of a protein (or proteins) that inhibits the transport of glucose into the cell [43,51]. In a similar vein, steroid-mediated synthesis of a specific protein may be required for the marked decrease in plasminogen activator activity observed in L cells exposed to dexamethasone. This requirement has not been directly determined in fibroblasts, but it may be inferred from the observation that the same dexamethasone-mediated decrease in plasminogen activator activity observed in cultured hepatoma cells is blocked by actinomycin D [20].

Although specific transport-inhibiting or other growth-inhibiting proteins have not been identified, there is abundant evidence of glucocorticoid-mediated enzyme induction in several cell types [1,53] and some evidence for enzyme induction in fibroblasts. The level of glutamine synthetase activity rises severalfold in L cells exposed to glucocorticoids [54], and the increase is blocked by both actinomycin D and cycloheximide [55]. Alkaline phosphatase activity increases in some cultures of skin fibroblasts exposed to glucocorticoids in vitro. This phenomenon was not observed in one study [56], but other investigators have found significant increases in alkaline phosphatase activity in human diploid skin fibroblasts after exposure to low concentrations of hormone [57,58].

In the case of alkaline phosphatase, it has not been established that the glucocorticoid-mediated rise of activity in fibroblasts is a consequence of new protein synthesis. Induction of alkaline phosphatase was carefully examined in a human epithelial cell line (HeLa 65 cells), and it is clear that in this case the increased level of activity was due to an increase in catalytic efficiency rather than to an increase in the amount of enzyme protein [59]. The hormone-mediated rise in enzyme activity in HeLa cells was prevented by actinomycin D and cycloheximide [59,60], a fact that suggests new protein synthesis is required. Alkaline phosphatase is a membrane-associated phosphoprotein. It has been purified from both control and glucocorticoid-treated HeLa cultures, and the enzyme obtained from the treated cells contains approximately one-half the phosphate residues associated with the control cell enzyme [61]. Thus, it would seem that in this cell type the glucocorticoids either induce the synthesis of a protein that alters a protein kinase activity or mediate the production of a specific protein phosphatase. In any event, a less phosphorylated alkaline phosphatase is produced and possibly because of this fact the molecule has increased catalytic activity. I have presented this example here for 2 reasons: (1) because a similar mechanism may operate in the glucocorticoid-mediated rise in alkaline phosphatase activity observed in human skin fibroblasts and (2) to emphasize the point that inhibition of a hormone-mediated increase in enzyme activity by inhibitors of RNA and protein synthesis should not lead one to conclude automatically that the hormone has induced the synthesis of that enzyme protein.

The possibility exists that modifications similar to those described for alkaline phosphatase in HeLa cells occur with molecules involved in transport or other cell functions. Perhaps then, instead of conceiving that glucocorticoids cause the induction of proteins that specifically inhibit selected biochemical processes, one should consider the possibility that they induce the synthesis of enzymes that modify proteins involved in key processes such as transport. One could hypothesize that the glucocorticoid-mediated induction of only a few enzymes capable of exerting such posttranslational modification of proteins could ultimately lead to growth inhibition. Phosphatases are reasonable candidates for the proposed induced enzymes because their effects could ramify throughout the system, and thus perhaps lead to decreased ATP levels, hexose and amino acid transport, and nucleic acid synthesis, all of which have been observed in cells in which glucocorticoids have a growth-inhibiting or killing effect.

The induction of a protein (or set of proteins) is suggested by experiments concerned with the effect of glucocorticoids on virus replication in fibroblasts. Dexamethasone and other glucocorticoids have been shown to stimulate virus production in mouse 3T3 fibroblasts infected with polyoma virus [62]. The stimulation of virus production is stereospecific and receptor-mediated. Also, the production of type-C virus induced by 5-iodo-2′-deoxyuridine (IUdR) in nonvirus-producing BALB/K3T3 mouse fibroblasts (previously infected with Kirsten murine sarcoma virus [Ki-MuSV]) is stimulated by glucocorticoids [63,64]. The steroid alone does not cause virus production in nonvirus-producing cells previously infected with Ki-MuSV. Rather, it enhances virus production in cells already induced with IUdR to produce virus [64]. The step affected by the hormone occurs late in the viral replication cycle, but the steroid apparently does not enhance virion assembly or release. Pretreatment of induced cells with low concentrations of cordycepin abolishes the dexamethasone stimulation, a fact that suggests the steroid-mediated effect comes after the addition of poly(A) to viral RNA [64]. These observations are consistent with the proposal that glucocorticoids induce the formation of a protein (or proteins) that affects virus-specific RNA translation. Wu et al. have tentatively concluded that the association of free virus-specific mRNA with ribosomes or the initiation of polypeptide chain synthesis may be enhanced [64].

Before terminating this discussion of potential gene products induced by glucocorticoids in fibroblasts, I should mention the negative results obtained in cell hybridization experiments. Several laboratories have reported the successful production of cell hybrids formed from cultured mouse fibroblasts (either L cell or 3T3 lines) and rat hepatoma (HTC) cells. Glucocorticoids cause the induction of high levels of tyrosine aminotransferase activity in HTC cells. The induction process has been studied in detail in the hepatoma cells [53], and it is clear that the steroid increases the rate of enzyme synthesis. Even though it could be shown that the hybrid cells contained elements of both the rat liver cell and mouse fibroblast genomes, the levels of tyrosine aminotransferase were low and the enzyme was not inducible by glucocorticoid [65–68].

Summary. From observations made in a variety of experimental systems, a model of glucocorticoid action has emerged (Fig 2). The essential features of the model apply for all of the steroid hormones. A critical concept is that the physiological effects of steroid action are mediated via the synthesis of specific mRNAs and proteins. The key induced proteins that are ultimately responsible for inhibition of fibroblast growth have not yet been identified, and their existence can only be inferred from indirect observations made in other cell types. It is probable that growth inhibition results from the production of only a very small number of proteins. Some of these induced molecules may be enzymes that by virtue of their effect on other key cellular components (protein components of transport systems, etc.) are able to compromise multiple systems and produce growth inhibition. The pleiotropic nature of glucocorticoid action in general could reflect the induction of a very few gene products, some of which are capable of exerting a posttranslational modification.

Interaction of Glucocorticoids with Receptors in Fibroblasts

A central concept in the model of steroid action is that the hormone must bind to a specific receptor protein in order to exert its effect on the cell. In the absence of steroid, the receptor is located in the cytoplasm. Thus, in contrast to neurotransmitters and polypeptide hormones, steroids must pass through the cell membrane before they can bind to the receptor. Glucocorticoids apparently traverse the cell membrane by passive diffusion, and, given the lipophilic nature of these compounds, equilibration across membranes is in general very rapid. A notable exception to this statement is the passage of glucocor-

ticoids across the epidermal barrier, where there is considerable impedence to penetration. In cultured mouse cell systems we have found that the concentration of free steroid achieved in the cytoplasm at equilibrium is not necessarily the same as that in the growth medium. Mouse fibroblasts (L cells), as well as lymphoma (ML-388) and adrenal (Y-1290S3) cells, possess a transport system that is capable of transporting some biologically active glucocorticoids, notably cortisol and dexamethasone, from the inside of the cell to the exterior [69,70]. We have not found this transport in two varieties of cells cultured from human sources (HeLa and WI-38 fibroblasts). The transportability of a steroid is unrelated to its biological activity. Tiamcinolone acetonide and dexamethasone, for example, are both biologically active glucocorticoids, but only the latter is transported. The biological role of the transport system has not been defined, but, since it keeps the concentrations of free cortisol and dexamethasone in the cytoplasm lower than those in the medium, these drugs appear somewhat weaker than they really are when their physiological effect in L cells is plotted against the concentration of drug added to the culture medium.

Co-workers and I were able to measure the binding of glucocorticoids to the ctoplasmic receptor in both intact fibroblasts [71] and in high-speed supernatants prepared from hypotonically ruptured cells [72]. The binding of triamcinolone acetonide to the L fibroblast receptor is presented in Fig 3 [73]. In this experiment, we incubated aliquots of a 100,000 ×g supernatant prepared from L cells with various concentrations of radiolabeled triamcinolone acetonide at 0°C for a sufficient time to permit the binding reaction to approach completion. Other samples of the supernatant were incubated with both radiolabeled triamcinolone acetonide and a high concentration (2×10^{-5}M) of nonradioactive dexamethasone. Each aliquot was then filtered through a small Sephadex G-25 column for the purpose of separating the bound from the free drug. The assay was based on the principle that the biologically active nonradioactive steroid would compete for association with specific receptor sites and prevent binding of the radiolabeled drug. No competition should be observed when the radiolabeled drug binds nonspecifically to other proteins in the solution. When the nonspecific binding is subtracted from the total binding, the difference represents binding to the specific receptor site (*dashed line*). It is evident that specific binding is of a high affinity and is saturable. A single set of binding sites is implied by the linear nature of the Scatchard plot presented in the inset.

There is considerable evidence that the observed binding represents the association of glucocorticoid with its receptor site. First, the binding was stereospecific. Only biologically active glucocorticoids and glucocorticoid antagonists (e.g., cortexolone) competed for the binding of radiolabeled triamcinolone acetonide to the fibroblast receptor [71,72]. Second, the binding affinity of different steroids reflected their physiological potency as inhibitors of L cell growth. This fact is shown in Fig 4, in which dose response curves of growth inhibition (shown at the top) are compared to specific binding data for three steroids (bottom half) dermined by direct assay as in the experiment summarized in Fig 3 [73]. Triamcinolone acetonide was the most potent of the steroids assayed; dexamethasone was of

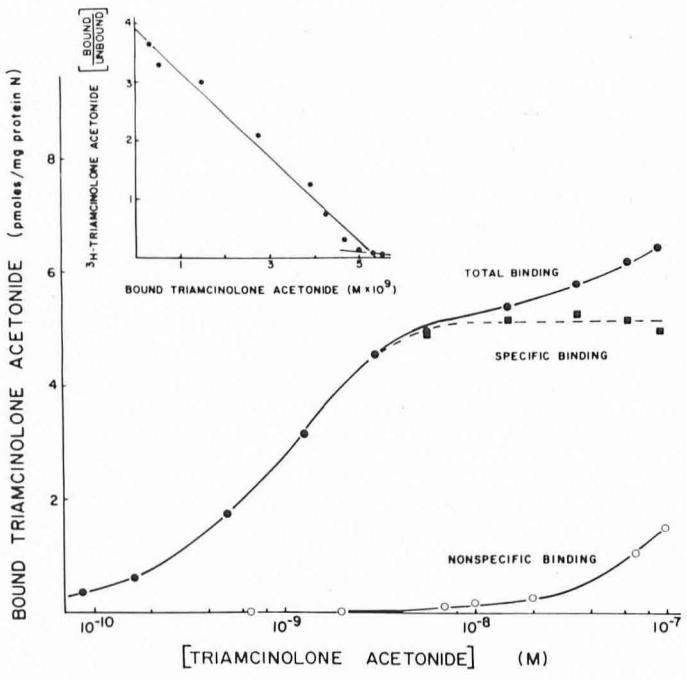

FIG 3. Specific binding of radiolabeled triamcinolone acetonide to the glucocorticoid receptor in L cell cytosol. Aliquots of a 100,000 ×g supernatant from L cells were incubated for 22 hr at 0°C with various concentrations of [^{3}H]triamcinolone acetonide in the presence (○) or absence (●) of 5×10^{-5} M nonradioactive dexamethasone. The bound radioactivity was assayed by passage of each incubation mixture through a short column of Sephadex G-25 and by determination of the radioactivity associated with the macromolecular fraction. Specific binding (■), represented by the *dashed line*, is the binding achieved in the absence of competing steroid (total binding) minus the binding in the presence of competitor (nonspecific binding). *Inset*: a Scatchard plot of the binding in the presence of vehicle (data from Pratt, Kaine, and Pratt [73]).

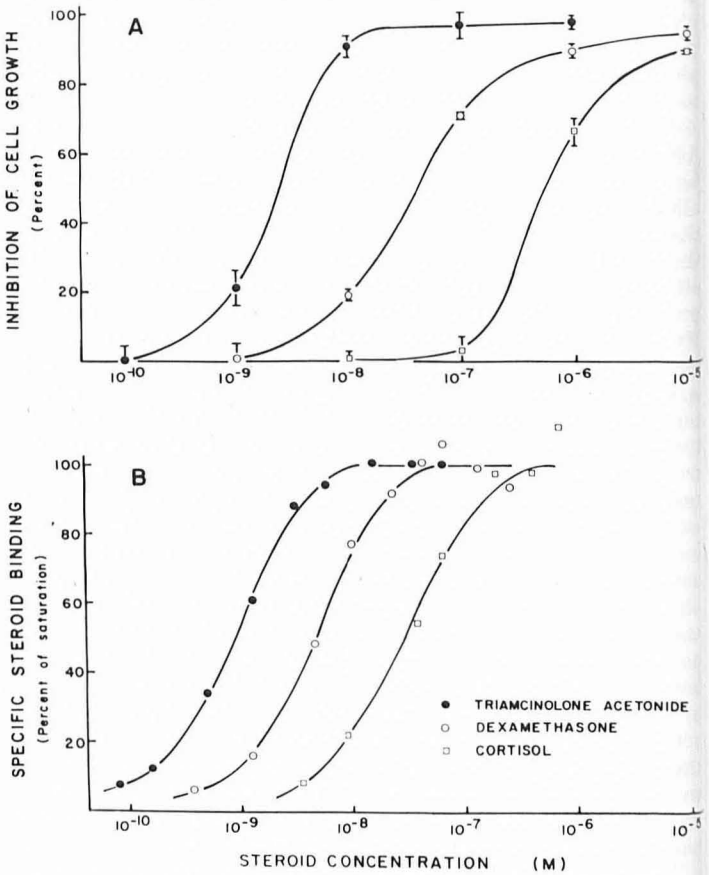

FIG 4. Comparison of the potency of triamcinolone acetonide, dexamethasone, and cortisol as inhibitors of L cell growth with the ability of each steroid to bind to the L cell receptor. The data of the top graph (*A*) are expressed as the percentage of inhibition of L cell growth obtained in 5 days of exposure to steroid as compared to inhibition in control cultures that grew in the presence of vehicle alone. "Steroid concentration" represents the amount of drug added to the culture medium. In the lower graph (*B*) the specific binding of each steroid, determined in cytosol preparations as described in Fig 3, is plotted as a percentage of the binding at saturation of the receptor versus the free concentration of steroid. ●, Triamcinolone acetonide; ○, dexamethasone; □, cortisol (data taken from Pratt, Kaine, and Pratt [73]).

intermediate potency and cortisol was the weakest. The direct binding curves are similar to the dose-response data with the restriction that the binding curves for cortisol and dexamethasone are shifted somewhat to the left of the growth-inhibition curves. This shift is probably a consequence of the fact that outward transport of these 2 steroids caused their intracellular concentrations to be lower than the concentration of drug added to the growth medium. Third, the most potent evidence that the binding represents association with the receptor site has been obtained from studies on resistant fibroblasts; L cells were grown in the presence of increasing concentrations of steroid until a cell population was obtained that was unresponsive to even the most potent glucocorticoids at concentrations a thousandfold higher than those that normally produce maximum growth inhibition in the parent line [71]. These cells were then cloned and shown to contain about 10% of the specific binding capacity of the sensitive fibroblasts [72]. Because there was no change in the affinity of the small amount of binding observed, the resistant cells must have lost the ability to bind the drug.

The binding of the drug to the receptor is presented in Fig 2 as a single-step bimolecular interaction (*equation 1,* Fig 5); however, observations suggest that the binding reaction is more complex. From a physicochemical standpoint, the rate of association of glucocorticoids with their receptors was very slow in all cell systems examined. In fibroblasts [72] the second order rate constants of association at 0°C were in the range of 3 to 7 $\times 10^5$ $M^{-1}min^{-1}$, and an appropriate description of the drug-receptor interaction must account for this very slow rate of binding. Also, an appropriate definition of the binding reaction must explain how drugs that bind to glucocorticoid receptors can act as agonists, partial agonists, or antagonists depending on their chemical structure. Two binding models have been advanced to explain these phenomena. In both cases, the assumption was made that the receptor is an allosteric system in which the steroid acts as an allosteric effector.

One proposal (*model 2* in Fig 5) assumes that the receptor equilibrates between 2 conformational forms, *x* and *y,* each of which has different activities with respect to enzyme induction [74,75]. In this scheme, the x form is the biologically active one. According to the model (originally proposed by Rubin and Changeux [76]), the behavior of the different classes of steroids is explained by their ability to determine different values of the active form as a result of their different affinities for the x and y forms. The model predicts that the inactive receptor conformation will predominate in the absence of steroid and that fully active glucocorticoids will bind principally to the active x form. In contrast, pure antagonists will bind only to the y form. The model provides an explanation for the slow binding rate as well as the agonistic, antagonistic, and partially agonistic effects.

1 $R + S \rightleftharpoons RS$

2 $R_X + S_{(agonist)} \rightleftharpoons R_X S$

$\downarrow\uparrow$

$R_Y + S_{(antagonist)} \rightleftharpoons R_Y S$

3 $R + S \overset{Fast}{\rightleftharpoons} (RS) \overset{Slow}{\rightleftharpoons} RS'$

FIG 5. Equations used to express various models of glucocorticoid binding. *1,* Single-step interaction. *2,* Two conformational state model [74]. Receptor equilibrates between 2 conformational forms, x and y. Agonists bind to the active form R_x and antagonists bind to the inactive form R_y produce biologically active and inactive complexes, respectively. *3,* Two-step model [73]. Both agonists and antagonists bind rapidly to the receptor site, but only agonists can participate in a second step in which the steroid-receptor complex is converted to a tight binding form, RS′.

We have proposed an alternative model consistent with our analysis of the kinetics of glucocorticoid binding to L 929 fibroblast receptors. This model is summarized by *reaction 3* in Fig 5 [73]; the steroid (regardless of whether it is an agonist, antagonist, or partial agonist) initially binds rapidly to form a weak complex with the specific binding site. This binding is too weak to be observed with standard assay procedures, but the weak complex formed with the biologically active steroids is slowly converted to the tight form observed in the assay. One can conceive that the pure antagonist participates in the initial weak interaction but that the antagonist-receptor complex cannot be converted to the tighter form needed for initiation of the physiological response. Partial agonists would produce an effect dependent on the proportion of receptor in the tight versus weak binding state at complete occupancy. Neither binding model has been proved true for glucocorticoids, although there is adequate precedent for both in other drug-receptor systems. I have presented the models here in order to point out that the conventional presentation of a simple single-step bimolecular interaction (*reaction 1*) does not explain all of the observations made in glucocorticoid binding systems.

Summary. In order to produce a physiological response, glucocorticoids must first bind to a receptor located in the cytosol fraction of the cell. The receptor binds glucocorticoids in a very specific manner. Only biologically active glucocorticoids and a few compounds that can act as glucocorticoid antagonists (antiglucocorticoids) can bind to the receptor site. The binding is reversible,* of high affinity, and saturable. The potency of glucocorticoids as inhibitors of fibroblast growth is directly related to the tightness of the binding complex that is formed with the receptor [73]. Some L cells that have been selected for glucocorticoid resistance contain only 10% of the specific binding capacity of the sensitive parent line. Two binding models have been presented (*models 2 and 3,* Fig 5) that are capable of explaining both the slow rate at which glucocorticoids form the binding complex and the fact that some steroids can act as partial agonists or antagonists. Two important assumptions I have made in considering the steroid receptor interaction are that the receptor is an allosteric system and that the steroid acts as an allosteric effector.

Composition and Physical Properties of the L Cell Receptor

All of the information available regarding the composition and properties of the L cell receptor was obtained from studies carried out on crude 100,000 ×g supernatant preparations. The binding of glucocorticoids was not affected by digestion with RNase, lipase, or neuraminidase, but it was eliminated by a variety of proteases [71,78]. The steroid-binding ability of the 100,000 ×g preparation was also inactivated by digestion with phospholipases A and C [78]. The phospholipase A effect was examined in some detail and found to be specific for the action of that enzyme. Boiled phospholipase A preparations caused a calcium-dependent inactivation of the binding capacity that was blocked both by phospholipid and a substrate analogue that competitively inhibits the enzyme [78]. One possible explanation for the phospholipase A effect is that the receptor protein may be associated in some way with a phospholipid component required for specific binding of the hormone. It is also possible that the action of phospholipase A on phospholipid

* We [Pratt and Ishii; 72] had originally thought that the binding of triamcinolone acetonide and other potent glucocorticoids to the L cell receptor was of the "pseudoirreversible" type observed, for example, between methotrexate and folate reductase at a low pH. This hypothesis clearly is not true, and subsequent studies by Krieger, Middlebrook, and Aronow [77] showed that we were misled by salt effects. The rate of inactivation of receptor by salt approaches the rate of dissociation; therefore, the effect of cold steroid chase, on which these conclusions were based was masked.

in cytosol preparations results in the production of lysophosphatides that inactivate the receptor through a detergent action. Other conditions that yield a loss of ability to bind steroid include exposure to solutions of high ionic strength [77], sulfhydryl-blocking reagents [79], and low concentrations of detergents of both denaturing and nondenaturing types.

Association with glucocorticoid markedly stabilized the receptor against inactivation [72]. Glucocorticoids stabilized the receptor roughly in proportion to their binding affinity [80]. Because of the lability of the unbound receptor, initial attempts at purification focused on use of conventional protein purification techniques to concentrate the bound radiolabeled triamcinolone acetonide-receptor complex [81]. This complex was purified about 2,000-fold, but the product was unstable and the ultimate yield was too low for measurement of its physical properties. The behavior of the unpurified triamcinolone acetonide-receptor complex was examined by Sephadex chromatography and sucrose gradient centrifugation in hypotonic buffer. It was nearly excluded from Sephadex G-200 (consistent with an apparent molecular weight of greater than 500,000), but the sedimentation coefficient (S20, *w*5.5) was consistent with the value expected for a globular protein with a molecular weight of about 85,000. This kind of discrepancy could be due to aggregation, association of the drug-receptor complex with nonprotein cellular components such as phospholipid, or extreme molecular asymmetry.

Middlebrook and Aronow [82] recently examined the physical properties of the drug-receptor complex in detail. It is clear from their studies that the physical behavior of the receptor in L cell cytosol differs markedly according to the salt concentration of the buffers employed. They determined a partial specific volume for the triamcinolone acetonide-receptor complex of 0.73 to 0.74. This is consistent with a molecule of solely protein composition and argues strongly against association of phospholipid with the hormone-receptor complex. Because the apparent size of the complex on Sephadex chromatography and sucrose gradient centrifugation varied widely according to the ionic strength, calculations of Stokes radii and molecular weights also varied widely. From their measurements, Middlebrook and Aronow concluded that the receptor is a fibrous protein with an axial ratio in isotonic buffer of 1:19 oblate or 1:14 prolate [82]. These values are so large, however, as to seem beyond the accuracy of such calculations; they may have been considerably distorted by difficulties with aggregation, which have been experienced by others who have studied the hepatic glucocorticoid receptor [83].

Several important observations made by Middlebrook and Aronow [82] suggest that there may be different forms of the hormone-receptor complex, both before and after association with the nucleus. The cytosol steroid-receptor complex was found to resolve into 3 peaks on isoelectric focusing (pK 5.8, 6.4, 6.8). It is possible that these peaks reflect forms of the cytosol complex existing before and after transformation to the nuclear bindable state. This possibility has been suggested for the glucocorticoid receptor in hepatic cytosol [84]. It is also possible that, as noted with progesterone-binding molecules, there is more than 1 binding protein with the same specificity and affinity for glucocorticoids. A third possibility is that the drug-receptor complex associates with other components in cytosol that alter its isoelectric point. An important observation made in this work is that the triamcinolone acetonide-receptor complex isolated by salt extraction of nuclei obtained from cells that had been incubated at 37°C with radiolabeled steroid (conditions appropriate for transformation and nuclear binding) had considerably different physical properties from those of the cytosol steroid-receptor complex. The complex obtained from nuclei had a much lower apparent molecular weight on Sephadex G-200 with a Stokes radius of 32Å versus 50Å for the cytosol complex in isotonic salt. The calculated molecular weight for the nuclear extractable form of the hormone-receptor complex was 54,000 versus 109,000 for that of the cytosol preparation.

In addition to the steroid-receptor complex that was extracted into 0.3 M KCl, nuclei from cells incubated with triamcinolone acetonide at 37°C contained a significant portion of the specifically bound drug in a form that was not extractable with salt-containing buffer [85]. This tightly bound form of the hormone-receptor complex has been called the nuclear residual form, and it can be released from crude chromatin preparations with DNase [85]. Although the sedimentation and gel filtration properties of this tightly bound complex could not be determined, it was found to be more stable to thermal inactivation than either the cytoplasmic form of the receptor prior to transformation to the nuclear bindable state or transformed receptor that was loosely associated with nuclei and extractable with 0.3 M KCl [82].

Summary. The glucocorticoid receptor in L cell cytosol appears to be a high-molecular-weight protein, and its physical behavior varies markedly according to the salt concentration. The hormone-receptor complex is probably very asymmetrical, and free sulfhydryl groups are probably required for binding. The determination of a partial specific volume of 0.73 to 0.74 is not consistent with the presence of substantial amounts of either sugars or phospholipid in the steroid-bound complex. The only observation inconsistent with a solely protein composition is that the binding activity of cytosol is inactivated by phospholipase action, an inconsistency that could be explained by detergent effects. There is a possibility that more than one physical form of the receptor exists in the cytosol. The hormone-receptor complex apparently associates in 2 ways with the nucleus: in a loosely bound, salt-extractable state and in a tightly bound ("nuclear residual") form that may be in close association with DNA.

The Glucocorticoid Receptor Cycle

After hormone binding takes place in the cytoplasm, the glucocorticoid-receptor complex is transformed to a form (RS^n) that is then capable of associating with nuclear components (*step 3,* Fig 2). Very little is known about this process in L cells. When intact fibroblasts were incubated with radioisotope-labeled glucocorticoid at 0°C, most of the bound steroid was recovered in the cytosol, but when they were incubated at 37°C, there was a shift of the bound radioactivity into the nucleus [85]. The mechanism of the temperature-dependent transformation is not well understood. Studies on the analogous process as it occurs with estrogen-binding proteins show that transformation in that case is a second order event [86]. The work of Middlebrook and Aronow suggests that temperature-dependent transformation of the L cell steroid-receptor complex may be accompanied by changes in its physical characteristics [82]. In any event, the resulting complex became associated with nuclei where, as described above, 2 association states were evident [82,85].

No definitive studies on the nature of the nuclear acceptor sites have been carried out in fibroblasts. There is some suggestion that there may be a defined number of sites with which the RS^n complex can interact. Lippman and Thompson [87] have shown that L cell cytosol receptor bound with dexamethasone and activated under cell-free conditions binds to washed L cell nuclei at 20°C. The nuclear binding of the steroid-receptor complex under these cell-free conditions is saturable at about 2.5×10^{-9} M. (This concentration represents the concentration of specifically bound hormone based on the molecular weight of the steroid itself.) It is not at all clear, however, that the nuclear binding observed under these cell-free conditions represents a faithful reproduction of the events as they occur in intact cells. The results of Lippmann and Thompson [68,87] imply that the association has some specificity since nuclei appear to bind different glucocorticoid receptors at different acceptor sites. These investigators found that the L cell dexamethasone-receptor complex binds to a saturable number of sites in either fibroblast or HTC cell nuclei. After binding the homologus steroid-receptor complex to saturation, either

type of nucleus can then bind the heterologous complex, presumably to a second set of saturable sites. Lippman and Thompson also suggest that hybrid L/HTC cells may contain both receptors and both classes of nuclear acceptor sites [68]. Again, it is not possible to accurately assess the meaning of their observations without the assurance that the assay system reflects the proper nuclear binding events.

Some information is available that contributes to our concept of what may happen to the receptor after it has bound in the nucleus. The information was obtained in studies on the energy requirement for maintenance of specific binding in the intact cell. In the first study that successfully demonstrated glucocorticoid binding to specific receptors, Munck and Brinck-Johnsen observed that the amount of binding assayed in intact thymic lymphocytes at 37°C was critically dependent on the metabolic state of the cell [88]. Specific steroid binding disappeared when thymocytes were incubated in glucose-free medium in an atmosphere of nitrogen, and it was rapidly restored when the gas phase was returned to oxygen. The cells regained their specific binding capacity even in the presence of cycloheximide [89]. Munck and his co-workers have proposed that the glucocorticoid receptor in thymic lymphocytes exists in 2 forms, and that energy (ATP?) is required to generate the steroid-binding form from a nonbinding precursor [89,90].

A similar energy requirement has been found in L 929 fibroblasts. When intact L cells were incubated with radiolabeled triamcinolone acetonide in glucose-free medium in the presence of dinitrophenol, there was a marked loss of specifically bound steroid from the cytosol (Fig 6) [91]. When cells were returned to a dinitrophenol-free, glucose-containing medium, steroid binding returned to control levels, a return that required energy and was not affected by inhibitors of protein synthesis. About one-third of the dinitrophenol-mediated disappearance of binding from the cytosol could be accounted for by increased recovery of specifically bound steroid in a low-speed (7,000 ×g) particulate fraction containing the L cell nuclei [91]. A profound shift of the steroid receptor complex to the nuclear fraction was observed when metabolism was blocked with cyanide instead of dinitrophenol [85,91]. After addition of cyanide to a suspension of L cells equilibrated with radiolabeled triamcinolone acetonide, most of the steroid-receptor complex was recovered in the tightly bound nuclear form [85].

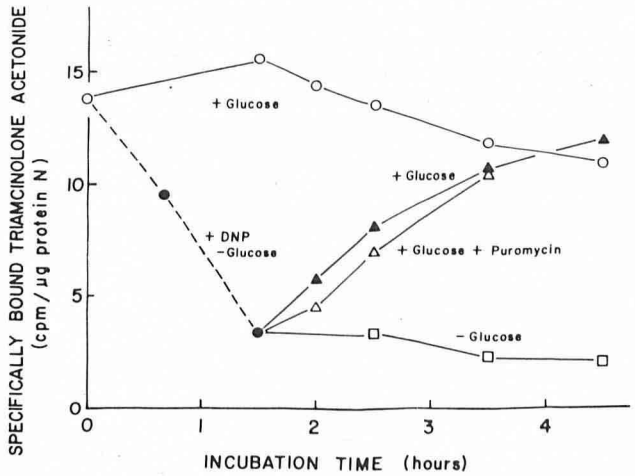

FIG 6. Reversible effect of a metabolic inhibitor on specific glucocorticoid binding in the soluble fraction of L cells. A suspension of L cells was preincubated for 1 hr at 37°C with radiolabeled triamcinolone acetonide. After the preincubation (0 time on the abscissa), the culture was split and a portion (●) was incubated in glucose-free medium containing 5×10^{-4} M dinitrophenol. After an additional 1.5 hr of incubation, the cells were again centrifuged, resuspended, and incubated at 37°C under the following conditions: (□), glucose-free medium; (▲), glucose-containing medium; (△), glucose-containing medium plus puromycin (0.2 mM). Controls (○) were submitted to all procedures, but resuspended in glucose-containing medium at 0 time and 1.5 hr. [^{3}H]Triamcinolone acetonide was present at 10 nM throughout the experiment. Each point represents the specifically bound triamcinolone acetonide assayed in a 7,000 ×g supernatant prepared from ruptured cells. The binding represents both cytoplasmic RS complex and the salt-extractable nuclear form (data abstracted from Ishii, Pratt, and Aronow [91]).

The observations made in fibroblasts appear to be most consistent with the existence of 2 energy-dependent steps (Fig 7) [92]. It seems reasonably clear that the receptor does not have to cycle through the nucleus in order for the steroid-binding form (R_a) to be inactivated to the nonsteroid-binding form (R_i) and for reactivation to occur. This statement is based upon 2 facts: (1) inactivation and activation of glucocorticoid-binding ability occurs in thymocytes incubated under the appropriate conditions in the absence of hormone [89] and (2) we have now carried out receptor inactivation and activation in isolated cytosol preparations from mouse fibroblasts (Sando and Pratt, work in progress).

The observation that a significant portion of the steroid-binding capacity of L cells became associated with the nucleus when the cell was exposed to metabolic inhibitors is consistent with the suggestion [91] that an energy-requiring process is required for release of receptor from the nuclear-bound form. There is, as yet, no evidence for such an energy-requiring release mechanism in other cell types. Sloman and Bell [93] carefully examined the effect of dinitrophenol on binding in thymic lymphocytes, and they found a reversible loss in cellular binding capacity but they did not observe an increased association of the steroid-receptor complex with the nuclear fraction. Munck et al. [90], from studies with intact rat thymocytes, and Ishii, Pratt, and Aronow [91], on the basis of observations made in mouse fibroblasts, have suggested that the recpetor is released from the nucleus in a form that cannot bind glucocorticoids (R_i).

Summary. It is clear that cells (thymocytes [88], fibroblasts [91], and chick embryo retina cells [94]) require energy in order to bind glucocorticoids in a normal manner. From observations made in several cell types it has been proposed [85,90–92] that the receptor is cycled through different states in the cell. It is possible that a single receptor protein may participate several times in the process of steroid binding and gene activation. The proposed receptor cycle may play an important role in determining the cell's ability to rapidly change the intensity of its physiological response in order to reflect constantly changing plasma glucocorticoid levels. The model of the receptor cycle presented in Fig 7 is clearly in an early stage of development, but it provides a

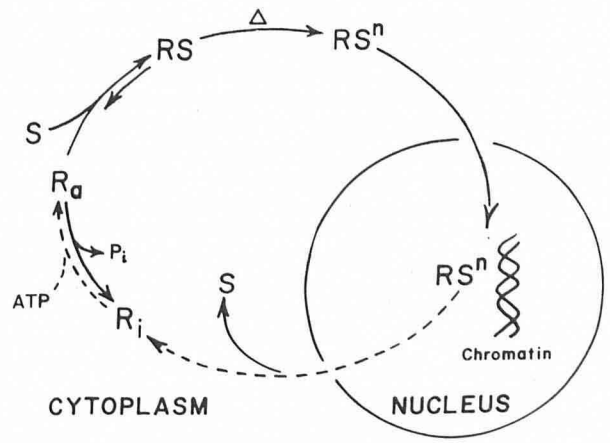

FIG 7. Proposed cycle of events controlling the binding state and cellular location of the glucocorticoid receptor. R_i, dephosphorylated form of the receptor that is inactive and cannot bind steroid; R_a, phosphorylated form of the receptor that binds glucocorticoid in the cytoplasm of the cell; *RS*, steroid-receptor complex; RS^n, form of the steroid-receptor complex that can bind to nuclear acceptor sites; *S*, steroid. *Broken lines* refer to processes that appear to require energy in L cells (taken from Nielsen, Sando, and Pratt [92]).

framework on which to ask a number of interesting questions regarding the molecular biology of glucocorticoid receptors.

Glucocorticoid Receptors and Fibroblast Response

The extent to which a cell can respond to glucocorticoids is directly proportional to its ability to bind the drugs. If the binding capacity of a cell is reduced, the physiological response, and consequently the clinical effect, is also reduced. All of the information available from both in vivo and in vitro observations is consistent with the proposal that glucocorticoid-responsive systems obey such an occupancy model.

There are now several well-studied examples of glucocorticoid resistance, and it is clear that in most cases the resistance is related to binding capacity. Sibley and Tomkins [95] isolated a number of glucocorticoid-resistant sublines of cultured murine lymphoma cells and determined the relationship between specific binding capacity and cell response. About 80% of their resistant cell lines were markedly deficient in binding capacity. The rest were equally divided between cells with normal cytoplasmic binding but with a deficiency in their ability to transfer the RS complex to the nucleus, and cells with normal binding and transfer that were apparently deficient at some step subsequent to the nuclear localization of the RS complex. Similarly, in studies on patients with acute lymphoblastic leukemia, a direct correlation was demonstrated between clinical response to glucocorticoid therapy and the specific binding capacity of isolated blast cells [96]. As mentioned previously, the same correlation between binding capacity and resistance has been demonstrated in L 929 fibroblasts [71,72]. An increased metabolism of steroids has also been suggested as a resistance mechanism in these cells, but the degree of biotransformation is not proportional to resistance and cells also become resistant to synthetic steroids that are not metabolized by this fibroblast line [97].

There are several ways in which the steroid-binding capacity of the cell might be altered. There may be a decreased amount of receptor protein in the partially responsive or nonresponsive cell, and this could result from either a decreased rate of receptor synthesis or an increased rate of receptor protein degradation. Alternatively, the amount of receptor protein may remain constant and the binding capacity may change as a result of a decreased rate of receptor activation or an increased rate of receptor inactivation. It has generally been assumed that a decreased glucocorticoid-binding capacity represents either an alteration in receptor affinity or a decrease in the amount of receptor protein. In the majority of cases no alteration in affinity has been found, and a decreased receptor protein has been assumed.

In order to better understand the mechanisms that determine cellular binding capacity, we examined the process of inactivation of fibroblast, thymocyte, and hepatic glucocorticoid receptors under cell-free conditions. Unoccupied glucocorticoid receptors become rapidly inactivated in cell-free preparations. In some instances they can be partially stabilized by sulfhydryl-protecting reagents such as dithiothreitol [98], but in most cases the basis for their lability has not been determined. It seemed possible to us that the loss of ability to bind glucocorticoids in cell-free systems might reflect the conversion of the receptor to a nonsteroid-binding form, as proposed from the observations made in intact cells. It is clear from our recent studies that the inactivation of glucocorticoid receptors as it occurs in cytosol preparations is due to a nonproteolytic enzymatic action [80,99]. In rat liver and thymocytes, a considerable amount of the inactivating enzyme was located in the 100,000 ×g pellet fraction, but there was moderate activity in high-speed supernatant as well. The distribution of the receptor-inactivating enzyme activity in mouse L cells was not determined. The receptor-inactivating enzyme was solubilized from the 100,000 ×g thymocyte pellet and partially purified. The solubilized enzyme inactivated the glucocorticoid-binding capacity in the 100,000 ×g supernatant from L fibroblasts, but it had no effect on the estradiol-binding capacity of a similar preparation from rat uterus [80]. The enzymes in liver, thymic lymphocytes, and fibroblasts inactivated unbound glucocorticoid receptors but they did not cause the release of steroid from the RS complex [80,99]. The inactivation of specific binding activity in mouse fibroblast or rat liver cytosol was inhibited by molybdate, fluoride, and glucose-1-phosphate. The solubilized enzyme from rat thymus was inhibited only by glucose-1-phosphate. These observations are consistent with the model presented in Fig 7, in which it is postulated that the inactivation process is due to dephosphorylation. It has not been shown that the receptor protein itself is the moiety that is dephosphorylated by the endogenous inactivating enzymes.

Additional information supporting the proposal that receptor inactivation is a consequence of dephosphorylation was obtained from experiments with purified alkaline phosphatase [92]. Highly purified alkaline phosphatase from calf intestine inactivated the glucocorticoid-binding capacity of high-speed soluble preparations from rat liver or mouse fibroblasts. The activity was clearly specific for phosphatase: (1) it was zinc-dependent, (2) the inactivation was blocked by arsenate, a competitive inhibitor of the enzyme, and (3) alkaline phosphatase activity and receptor-inactivating activity coeluted on DEAE-cellulose purification of the enzyme. The purified phosphatase inactivated unbound receptor but did not release steroid from the RS complex. In a mixed preparation containing both fibroblast glucocorticoid receptor and uterine estrogen receptor, the glucocorticoid-binding capacity was selectively inactivated.

We are just now beginning to investigate the receptor activation process in cell-free systems. After permitting the specific binding capacity of the 100,000 ×g supernatant from rat thymocytes to become inactivated by incubation at 20°C, we are able to reactivate it by addition of a factor (or factors) present in the high-speed supernatant of mouse fibroblasts [100]. We do not know how reactivation occurs in this thymocyte system, but Granberg and Ballard [101] found that a similar reactivation by liver cytosol is possibly due to maintenance of sulfhydryl groups in the reduced form. In L cells there is some suggestion that reactivation involves a phosphorylation mechanism [100]. The type of data that suggests the possibility of phosphoryla-

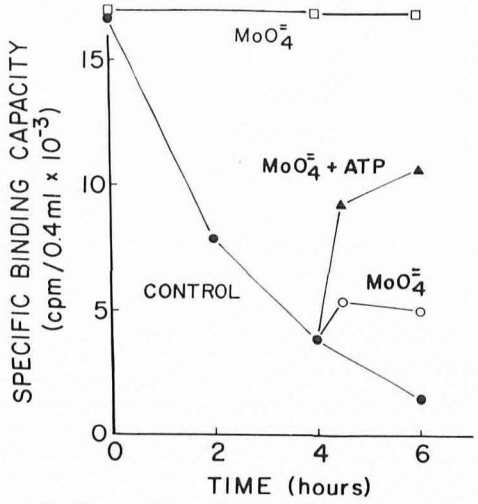

FIG 8. Inactivation and reactivation of the glucocorticoid-binding capacity of fibroblast cytosol. Aliquots of a 100,000 ×g supernatant from mouse L cells were incubated at 25°C in the presence (□) or absence (●) of 10 mM sodium molybdate, an inhibitor of phosphatase action. After 4 hr, either molybdate (○) or molybdate and 10 mM ATP (▲) were added to aliquots of control cytosol and the incubation was continued at 25°C. At each time point, samples were removed and specific binding capacity was assayed by incubation with [^{3}H]triamcinolone acetonide in the presence and absence of competing dexamethasone for 2 hr at 0°C (data taken from Sando and Pratt, work in progress).

tion is presented in Fig 8. In this case a 100,000 ×g supernatant from fibroblasts was incubated at 25°C in the presence or absence of molybdate, and at various times, aliquots were removed and assayed for their ability to bind radiolabeled glucocorticoid in a specific manner. The receptor activity of the control declined, whereas that in samples containing the inhibitor of phosphatase action remained stable. When further inactivation of the control was prevented by exposure to molybdate at 4 hr, addition of ATP permitted considerable reactivation of receptor activity. This ATP-dependent event may represent the same conversion of inactive to active receptor that has been proposed for the intact cell.

Summary. The extent to which a cell can respond to glucocorticoids is directly proportional to its ability to bind the drugs. If the binding capacity of the cell is reduced, the physiological response, and consequently the clinical effect, is also reduced. It has been shown that glucocorticoid resistance is most often associated with a decrease in (or complete loss of) receptor activity. The receptor activity of a cell could be altered either by affecting the amount of receptor protein or the proportion of the receptor that is in the active, steroid-binding state. From experiments demonstrating the energy requirement for maintenance of glucocorticoid receptor activity in intact cells, it has been proposed that receptor can be activated by a phosphorylation process and inactivated by dephosphorylation. Two observations made in cell-free preparations from fibroblasts and liver support the proposal that receptor activity is lost by a dephosphorylation process. First, the specific glucocorticoid-binding capacity was inactivated by alkaline phosphatase. Second, the receptor inactivation caused by endogenous enzymes was prevented by inhibitors of phosphatase action. The inactivation that occurred in cell-free preparations through endogenous enzyme action is reversible. Further study of the reactions affecting receptor activity may lay the foundation for clinical methods of drug treatment that shift greater amounts of receptor to the active form and enhance some glucocorticoid effects.

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Dermatology Seminar at Hawaii: 1979

The Third Annual Dermatology Seminar at Hawaii sponsored by the University of California, San Francisco, Cleveland Clinic and Northwestern University starts the evening of February 12th and ends approximately noon February 17, at the Hotel, Intercontinental, Maui, Kehei, Hawaii. The speakers include several guests and the faculty of the above departments. Enrollment is limited and made on a *first come first serve* basis. For information write the Department of Dermatology, at either University of California Medical School, San Francisco, California 94143, Cleveland Clinic, Cleveland, Ohio, or Northwestern University Medical School, Chicago, Ill.